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Soybean photosynthesis, Rubisco, and carbohydrate enzymes function at supraoptimal temperatures in elevated CO_2

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Summary

Soybean (*Glycine max* L. Merr. cv. Bragg) was grown season-long in eight sunlit, controlled-environment chambers at two daytime [CO_2] of 350 (ambient) and 700 (elevated) μmol mol^{-1}. Dry bulb day/night maximum/minimum air temperatures, which followed a continuously and diurnally varying, near sine-wave control set point that operated between maximum (daytime, at 1500 EST) and minimum (nighttime, at 0700 EST) values, were controlled at 28/18 and 40/30 °C for the ambient-CO_2 plants, and at 28/18, 32/22, 36/26, 40/30, 44/34 and 48/38 °C for the elevated-CO_2 plants. The objective was to assess the upper threshold tolerance of photosynthesis and carbohydrate metabolism with increasing temperatures at elevated [CO_2], as it is predicted that air temperatures could rise as much as 4–6 °C within the 21st century with a doubling of atmospheric [CO_2]. Leaf photosynthesis measured at growth [CO_2] and temperature was greater for elevated-CO_2 plants and was highest at 32/22 °C, but markedly declined at temperatures above 40/30 °C. Growth temperatures from 28/18 to 40/30 °C had little effect on midday total activity and protein content of Rubisco, while higher temperatures substantially reduced them. Conversely, midday Rubisco *rbcS* transcript abundance declined with increasing temperatures from 28/18 to 48/38 °C. Elevated-CO_2 plants exceeded the ambient-CO_2 plants in most aspects of carbohydrate metabolism. Under elevated [CO_2], midday activities of ADPG pyrophosphorylase and sucrose-P synthase and invertase paralleled net increases in starch and sucrose contents, respectively. They were highest at 36/26–40/30 °C, but declined at higher or lower growth temperatures. Thus, in the absence of other climatic stresses, soybean photosynthesis and carbohydrate metabolism would perform well under rising atmospheric [CO_2] and temperature predicted for the 21st century.

Key words: carbohydrate metabolism – CO_2 enrichment – *Glycine max* – high temperature – *rbcS* transcript – Rubisco – soybean

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Abbreviations: CER CO_2 exchange rate. – DAP days after planting. – EST eastern standard time. – PPFD photosynthetic photon flux density. – *rbcS* gene encoding for the small subunit of Rubisco. – Rubisco ribulose bisphosphate carboxylase–oxygenase. – SPS sucrose-P synthase. – VPD vapor pressure deficit

Introduction

The current atmospheric CO_2 concentration ([CO_2]) limits the photosynthetic capability, growth and productivity of many agricultural crop plants, among which the C_3 species show the greatest potential for response to rising [CO_2] (Bowes 1993, Kimball et al. 1993, Allen 1994, Drake et al. 1997). In a leaf, the photosynthetic CO_2 exchange rate (CER) is directly influenced by the activity of Rubisco, which in turn is influenced by various environmental factors, including atmospheric [CO_2] and air temperature. Furthermore, accumulation of the primary photosynthetic products, i.e. starch and sucrose, and activities of the key enzymes responsible for their metabolism, are undoubtedly under control and/or regulation by aerial environmental conditions. For many plants, elevated growth [CO_2] often results in photosynthetic acclimation characterized by a reduction in Rubisco protein concentration (Bowes 1993, Drake et al. 1997). In addition, decreased expression of the gene encoding the small subunit of Rubisco has been implicated in the down-regulation of photosynthesis under elevated [CO_2] (Van Oosten and Besford 1994, Van Oosten et al. 1994, Nie et al. 1995, Cheng et al. 1998, Gesch et al. 1998, Moore et al. 1998, 1999, Vu et al. 1999). Besides elevated CO_2, it has been suggested that plant growth under temperature stress may also lead to increases in leaf carbohydrates which may in turn repress the expression of *rbcS* genes (Webber et al. 1994). Increased carbohydrate content per se, however, does not directly inhibit the expression of such genes, but instead appears to be associated with the metabolism of hexoses and may be enhanced via sucrose hydrolysis by acid invertase (Goldschmidt and Huber 1992, Krapp et al. 1993, Moore et al. 1998). As a consequence of rising [CO_2] and other "greenhouse" gases (King et al. 1992, Keeling et al. 1995), atmospheric general circulation models predict significant increases in global air temperatures, possibly as much as 4 to 6 °C (Wilson and Mitchell 1987, Hansen et al. 1988, Kattenberg et al. 1996). Presently however, there is little if any information that exists describing the metabolism of starch and sucrose and the expression of photosynthetic genes during long-term growth of plants under both elevated [CO_2] and temperature.

Most studies evaluating the response of soybean growth and photosynthesis to elevated [CO_2] and temperature have been conducted at temperatures lower than 36 °C (Thomas et al. 1981, Sionit et al. 1987, Baker et al. 1989, Campbell et al. 1990). Ziska and Bunce (1997) measured the CER and biomass accumulation of soybean grown under ambient and double-ambient [CO_2] at constant day/night temperatures up to 40 °C in artificial-light growth chambers. Their experiments, however, were carried out only for three weeks from seed planting, and soybean plants were still in the developing seedling stage at the final sampling. In a previous study, we cultivated soybean to full maturity under natural sunlight at ambient and twice-ambient [CO_2] and under maximum daytime temperature regimes ranging from 28 to 40 °C, but were unable to assess an upper temperature threshold for leaf photosynthesis under elevated [CO_2] (Vu et al. 1997).

In this study, soybean was grown to full maturity at ambient and twice-ambient CO_2 and under day/night maximum/minimum air temperatures from 28/18 to 48/38 °C. Our objectives were to assess the upper threshold of soybean leaf photosynthesis and carbohydrate metabolism with rising air temperature and atmospheric CO_2, and to test the hypothesis that activities of the key enzymes for starch and sucrose metabolism in soybean leaves might be up-regulated under elevated growth CO_2 and temperature. A further objective was to test whether increasing growth temperatures altered the genetic expression of *rbcS* in soybean.

Materials and Methods

Plant material and growth conditions

Soybean (*Glycine max* L. Merr. cv. Bragg) was grown in eight sunlit, controlled-environment growth chambers located outdoors in Gainesville, Florida, as reported previously (Vu et al. 1997). Seeds were first inoculated with *Rhizobium* and were then planted on 11 February 1994 in 32-cm rows, resulting in a total of 40 plants m^{-2} at mid-growth season. Shades made of black, densely-woven, polypropylene fibers were maintained at canopy height to provide a light environment similar to that created by border rows in a field crop. The soybean plants were grown throughout their life cycle at two daytime [CO_2] of 350 (two chambers) and 700 (six chambers) µmol mol^{-1} air. Nighttime [CO_2] in each chamber was monitored and controlled to near ambient through a venting procedure (Baker et al. 1997b). This was done for 13 min at hourly intervals at night by automatically venting and flushing the chambers with ambient air while the air vent gates were opened and resealing the chambers, and the rise in chamber [CO_2] due to plant canopy respiration was monitored. Dry bulb day/night maximum/minimum air temperatures were controlled at 28/18 and 40/30 °C for the two chambers maintained at 350 µmol CO_2 mol^{-1}, and at 28/18, 32/22, 36/26, 40/30, 44/34 and 48/38 °C for the six chambers maintained at 700 µmol CO_2 mol^{-1}. The dry bulb air temperatures followed a modified sinusoidal control set point that varied continuously between maximum (daytime, at 1500 EST) and minimum

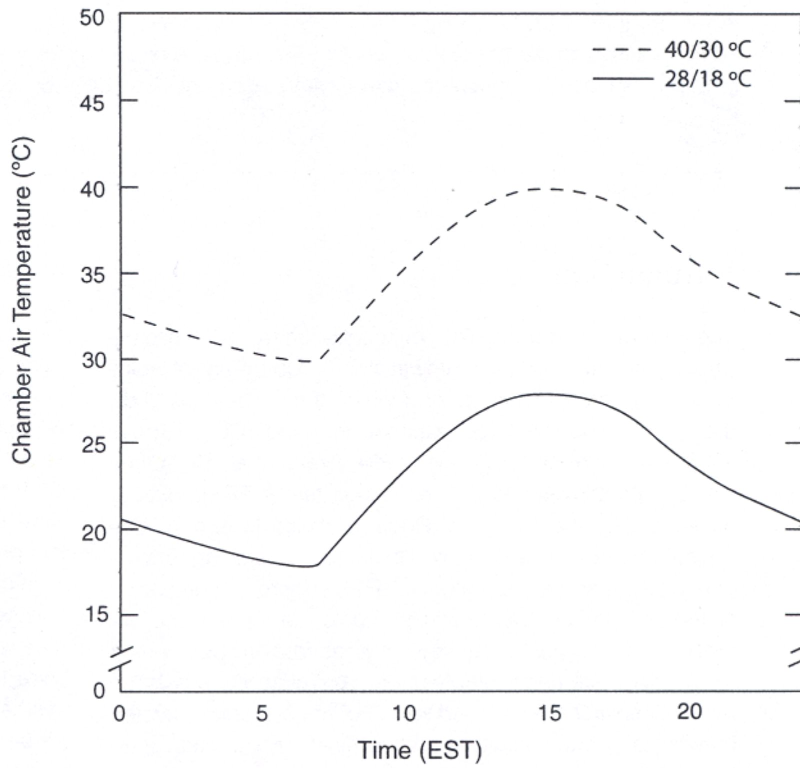

Figure 1. Example of the quality of dry bulb air temperature controls for the 28/18 and 40/30 °C regimes. Chamber dry bulb air temperatures were controlled to follow a continuously and diurnally varying, near sine-wave control setpoint that operated between maximum (daytime, at 1500 EST) and minimum (nighttime, at 0700 EST) values.

(nighttime, at 0700 EST) values (Figure 1). Dewpoints were controlled with the same type of modified sinusoidal pattern with daily maximum/minimum temperatures of 12/10, 16/12, 20/14, 24/16, 28/18, and 32/20 °C that corresponded to the six air temperature patterns. These diurnal dry bulb air temperature and dewpoint temperature controls led to the corresponding vapor pressure deficits (VPD) of 2.4/0.8, 2.9/1.2, 3.6/1.8, 4.4/2.4, 5.3/3.3, and 6.4/4.3, respectively.

Although the experimental design used for this study did not allow for the replication of chambers, the within-chamber estimates of variances were, however, similar to those measured between replicated chambers, as previous experiments at this location have shown (Baker et al. 1997a). The detailed chamber characteristics, specific methods for chamber environmental controls for temperature and [CO_2], and the quality of these controls are described by Jones et al. (1984), Pickering et al. (1994), and Baker et al. (1990, 1997a, b).

Leaf photosynthesis measurements

CER of single, attached, fully eypanded sun leaves was measured at midday, between 1100 and 1400 eastern standard time (EST), when solar PPFD was saturating at 1,400–1,800 µmol m^{-2} s^{-1}, using the LI-COR LI-6200 Portable Photosynthesis System and LI 6000-10 (4-dm^3 volume) cuvette (Vu et al. 1997). Measurements were made between 48 and 54 days after planting (DAP).

Leaf sampling for biochemical analyses

At 48 DAP, sampling of leaves for each [CO_2] and temperature treatment was performed before sunrise (0515 to 0545 EST, solar PPFD = 0), and near midday (1330 to 1400 EST, solar PPFD ~ 1,700 µmol m^{-2} s^{-1}). At each sampling time, ten uppermost fully expanded leaves were detached from ten different plants for each treatment and immediately immersed in liquid N_2. For the 48/38 °C growth at 700 µmol CO_2 mol^{-1}, only five uppermost fully expanded leaves were sampled at each sampling time, since only about one-fourth of the plants in this treatment (mainly those growing near chamber end walls) survived to this growth stage. Sampled leaves were pooled by treatment, ground to a fine powder in liquid N_2 with a mortar and pestle, and stored in liquid N_2 until analysis. Leaf fresh weight and area were also determined for a subset of plants at the same time of leaf sampling for biochemical analyses.

Determination of Rubisco activity and content

Rubisco total activity was measured for midday-sampled leaves by a modification of the procedure previously reported (Vu et al. 1997). A portion of the leaf frozen powder, about 0.3 g, was transferred to a pre-chilled Ten Broeck homogenizer and was ground at 2 °C in 4 mL of extraction medium consisting of 50 mmol/L Bicine-NaOH (pH 8.0), 10 mmol/L $MgCl_2$, 5 mmol/L DTT, 10 mmol/L D-isoascorbate, 0.1 mmol/L EDTA-Na_2, and 2 % (w/v) PVP-40. The homogenate was micro-centrifuged at 12,000 × g for 45 s at 2 °C, and an aliquot of the supernatant was immediately assayed for Rubisco activity. In addition, a 0.25-mL aliquot was frozen and stored in liquid N_2 for later analysis of Rubisco protein content. Rubisco total activity was measured by injecting 0.1 mL of the supernatant into 0.4 mL of an assay mixture consisting of 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L DTT, 10 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, and 20 mmol/L $NaH^{14}CO_3$ (2.0 GBq $mmol^{-1}$) at

30 °C. After a 5-min activation period, the reaction was initiated by adding RuBP to 0.5 mmol/L and terminating after 30 s with 0.1 mL of 6 mol/L HCl. After assay, the mixtures were dried at 60 °C and the acid-stable ^{14}C radioactivity was determined by liquid scintillation spectrometry.

Rubisco protein content was determined by a modification of the radio-immuno precipitation procedure previously reported (Vu et al. 1997). To the 0.25-mL leaf extract aliquot, $NaHCO_3$ was added to 10 mmol/L and the mixture was incubated at 25 °C for 5 min to activate Rubisco. A 20-µL aliquot of the mixture was then added to 50 µL of buffer (100 mmol/L Bicine, 20 mmol/L $MgCl_2$, 1 mmol/L EDTA at pH 7.8) containing 4 nmol of [2-^{14}C]-carboxyarabinitol bisphosphate (1.92 TBq mol^{-1}) and 50 µL of antiserum to purified tobacco Rubisco raised from rabbits. After incubation for 2 h at 37 °C, the precipitate was collected on a Millipore cellulose acetate/nitrate filter (0.45-µm pore size) and washed with 5 mL of a 0.85 % (w/v) solution containing 10 mmol/L $MgCl_2$, and the bound ^{14}C was determined by liquid scintillation spectrometry.

Analysis of Rubisco transcript

Total RNA was isolated from 0.5 g liquid N_2-frozen midday-sampling leaf powder, using a single-step method (Puissant and Houdebine 1990). Individual RNA samples were scanned between 320 and 220 nm and quantified by their absorbance at 260 nm. Ten micrograms of RNA per sample were separated on denaturating agarose-formaldehyde gels and blotted to positively charged nylon membranes (Boehringer Mannheim Biochemicals) as described by Gesch et al. (1998). A duplicate set of samples was run on the same gel and stained with ethidium bromide (1 mg mL^{-1} stock solution) to verify integrity and loading of RNA.

Detection of *rbcS* mRNA was performed as described by Vu et al. (1999). Blots were stripped and reprobed with a digoxigenin (DIG)-labeled 18S rRNA probe (Nairn and Ferl 1988) which was used to standardize loading. The DIG label was detected by chemiluminescence (CSPD, Boehringer Mannheim Biochemicals) by exposing membranes to X-ray at room temperature. Signal strengths were quantified by image-density scanning (IS-1000, Alpha Innotech Corp., San Leaudro, CA) and normalized with respect to the amount of 18S rRNA in each lane. Northern analysis was performed at least twice for each sample.

Assays of ADPG pyrophosphorylase, sucrose-P synthase, and invertase

Portions of the liquid N_2-frozen leaf powder of midday and predawn samplings were extracted and assayed for activities of ADPG pyrophosphorylase, sucrose-P synthase (SPS), and invertase with some modifications of the procedures as reported by Nakamura et al. (1989), Huber et al. (1989), and Huber (1989), respectively. For measurement of ADPG pyrophosphorylase activity, about 0.2 g of liquid N_2-frozen leaf powder was ground in 3 mL of 50 mmol/L HEPES-NaOH buffer (pH 7.4) containing 10 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 5 mmol/L DTT and 10 % (v/v) glycerol. The homogenate was microcentrifuged at 12,000 × g for 2 min at 2 °C, and the supernatant was immediately assayed for ADPG pyrophosphorylase (Nakamura et al. 1989). The assay was initiated by incubating 20 µL of the leaf extract in a reaction mixture containing 100 mmol/L HEPES-NaOH (pH 7.4), 5 mmol/L $MgCl_2$, 4 mmol/L DTT, 3 mmol/L PGA, 3 mmol/L PP_i and 2 mmol/L ADPG in a total assay volume of 0.25 mL. After 10 min at 30 °C, the reaction was terminated by placing the tubes in boiling water for 1 min. Samples were diluted with 0.35 mL of deionized water and microcentrifuged at 12,000 × g for 5 min at 2 °C. A 0.5-mL aliquot of the supernatant was mixed with 15 µL of 10 mmol/L NADP, and the initial absorbance value at 340 nm was recorded. Phosphoglucomutase and G-6-P dehydrogenase (1 unit each) were then added to initiate the reaction, and increases in absorbancy at 340 nm were recorded.

For measurements of SPS and invertase, about 0.15 g leaf powder was ground in 2 mL of 50 mmol/L MOPS-NaOH buffer (pH 7.5) containing 15 mmol/L $MgCl_2$, 1 mmol/L EDTA, 2.5 mmol/L DTT and 0.1 % (v/v) Triton X-100. The homogenate was microcentrifuged at 12,000 × g for 1 min at 2 °C, and the supernatant was rapidly desalted by centrifugal filtration on Sephadex G-25 column. SPS was assayed under both saturating (V_{max}) and limiting (V_{lim}) substrate conditions as F-6-P-dependent formation of sucrose (+ sucrose-P) from UDPG (Huber et al. 1989). Under V_{max} conditions, 45 µL of the leaf extract was incubated in a reaction mixture containing 50 mmol/L MOPS-NaOH (pH 7.5), 15 mmol/L $MgCl_2$, 2.5 mmol/L DTT, 10 mmol/L UDPG, 10 mmol/L F-6-P and 40 mmol/L G-6-P in a total volume of 70 µL. Under V_{lim} conditions, everything was the same except that 10 mmol/L Pi (an inhibitor) was included, and concentrations of UDPG, F-6-P and G-6-P were reduced to 2.5, 2.5 and 10 mmol/L. Assay reactions were terminated after 10 min at 30 °C with 70 µL of 1 mol/L NaOH, and assay tubes were immersed in boiling water for 10 min to destroy all unreacted F-6-P. After cooling to room temperature, 0.5 mL of 0.1 % (w/v) resorcinol in 95 % ethanol and 1.5 mL of 30 % (v/v) HCl were sequentially added. The tubes were incubated at 80 °C for 8 min, cooled for 5 min in tap water, and absorbance was read at 520 nm. Blanks were run in parallel using the complete assay reaction mixture plus denatured enzyme.

Soluble invertase was assayed at 37 °C in a reaction mixture (200 µL total volume) containing 100 mmol/L citrate-phosphate buffer (pH 5.0) for acid invertase, or 100 mmol/L MOPS-NaOH buffer (pH 7.0) for neutral invertase, and 50 mmol/L sucrose (Huber 1989). Reactions were initiated by addition of 40 µL desalted enzyme. At 0, 15, and 30 min, aliquots were removed and heat-killed. Analysis of glucose plus fructose was performed using the microtiter procedures described by Cairns (1987) and Hendrix (1993).

Determination of leaf starch and soluble sugars

Approximately 0.1 g of liquid N_2-frozen leaf powder of midday and predawn samplings was extracted three times, each with 4 mL of 80 % (v/v) ethanol at 85 °C for 1 h. After centrifugation, the combined supernatants were brought to a volume of 15 mL with 80 % ethanol, treated with activated charcoal, and aliquots were assayed for glucose, fructose and sucrose (Cairns 1987, Hendrix 1993).

The pellets which contained starch were oven-dried overnight at 60 °C. Starch in the pellet was first gelatinized by addition of 1 mL of 0.2 mol/L KOH and incubation in a boiling water bath for 30 min (Rufty and Huber 1983). After cooling to room temperature, 0.2 mL of 1 mol/L acetic acid was added, and the solution was incubated with 2 mL acetate buffer (pH 4.6) containing amyloglucosidase (6 units, Boehringer Mannheim) at 55 °C for 1 h. The reaction was then terminated in a boiling water bath for 5 min, and the resulting supernatant was analyzed for glucose (Cairns 1987, Hendrix 1993).

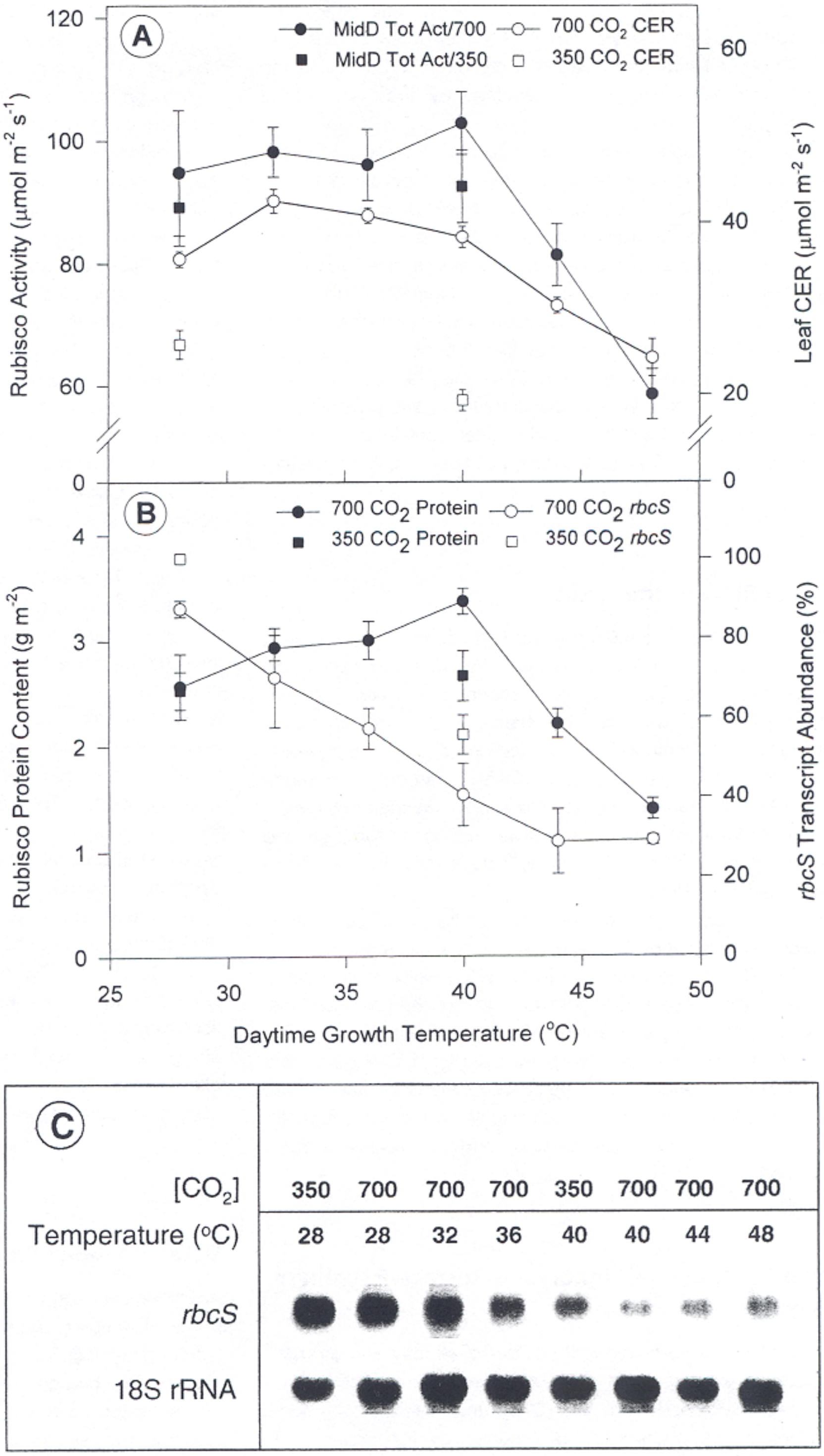

Figure 2. (A) Leaf photosynthetic rates and total activities of Rubisco, **(B)** Rubisco protein content and relative *rbcS* transcript abundance, and **(C)** Northern-blot analysis of *rbcS* transcript abundance of soybeans grown under 350 and 700 µmol CO_2 mol^{-1} and various temperature regimes. Measurements of leaf photosynthesis were made on uppermost fully expanded leaves 48 to 54 days after planting (DAP). Photosynthetic photon flux density during measurements, from 1100 to 1400 EDT, was 1400–1800 µmol m^{-2} s^{-1}. Rubisco measurements were performed on uppermost fully expanded leaves harvested at midday (PPFD ~ 1,700 µmol m^{-2} s^{-1}) 48 DAP. Rubisco activities **(A)** and protein content **(B)** were expressed on a leaf area basis; *rbcS* transcript **(B)** was expressed relative to the abundance in the 350 µmol CO_2 mol^{-1} plants at 28 °C; signal strengths of *rbcS* **(C)** were normalized with respect to the amount of 18S rRNA. The temperatures shown are daytime maximum air temperatures. Each data point for leaf photosynthetic rates represents the mean (with SE bar) of 5 to 23 observations. For Rubisco, each data point represents the mean (with SE bar) of 3 determinations. Where no bar is visible, the SE is smaller than the symbol.

Results

Soybean leaf CER, when measured at the growth [CO_2], was substantially enhanced by elevated CO_2, and the degree of enhancement increased with temperature (Figure 2A). Thus at 40 °C and 700 µmol CO_2 mol^{-1}, the leaf CER was double that of the ambient-CO_2 control. In terms of response to growth temperature, the leaf CER of plants in the elevated-CO_2 treatment showed an optimum at 32 °C, but the rate remained between 36 to 42 µmol m^{-2} leaf area s^{-1} over a wide temperature range (i.e. 28 to 40 °C). Even at the extreme growth temperature (48 °C), photosynthesis still functioned at

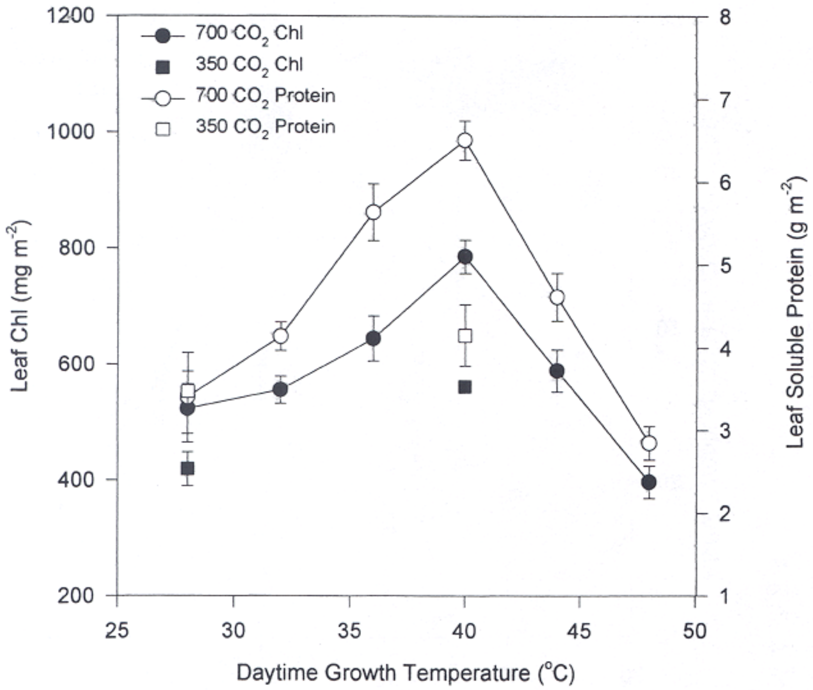

Figure 3. Leaf chlorophyll and soluble protein contents in midday-sampled leaves of soybean plants grown under 350 and 700 μmol CO_2 mol^{-1} and various temperature regimes. Uppermost fully expanded leaves were harvested for analysis at 48 DAP. Total chlorophyll and protein contents were expressed on a leaf area basis. Each data point represents the mean (with SE bar) of 3 determinations. Where no bar is visible, the SE is smaller than the symbol.

more than half of its optimum temperature rate. High growth temperatures appeared to be more deleterious to the ambient-CO_2 grown plants, as the CER values at 40 °C were 25 % less than at 28 °C. A relationship between chamber air temperature and leaf temperature, $T_{leaf} = 15.1 + 0.5T_{air}$, which was obtained by measuring the foliage temperature with infrared radiation thermometer in each chamber at midday of 35 DAP (Pan 1996), showed that leaf temperature was progressively lower than air temperature as air temperature increased above 30 °C. A 12 °C rise in air temperature, from 32 to 44 °C, was accompanied by only a 6 °C rise in leaf temperature, from 31.1 to 37.1 °C, respectively.

Total Rubisco activity of leaves sampled at midday for soybeans grown at ambient and elevated [CO_2] and several temperature regimes is also shown in Figure 2 A. There was no evidence that growth at elevated CO_2 resulted in down-regulation of midday total Rubisco activity, expressed on a leaf area basis. If anything, the rates were slightly higher than in leaves from the ambient-CO_2 grown plants (about 6 and 11 % higher at 28 and 40 °C, respectively). Midday total Rubisco activity remained high at growth temperatures between 28 and 40 °C, irrespective of the growth [CO_2]. Above 40 °C, the activity declined, dropping by about 40 % at 48 °C in elevated [CO_2]-grown plants.

As shown in Figure 2 B, Rubisco protein content in midday-sampled leaves followed a similar trend to that of the enzyme activity. Thus, it was the same or slightly higher in elevated CO_2-grown plants, and showed no decrease with increasing growth temperature up to 40 °C, but declined thereafter, by about 60 % at 48 °C.

The data for *rbcS* transcript abundance were in marked contrast to those for Rubisco activity or protein content as Figure 2 B and C demonstrates. Midday-sampled leaves from the elevated-CO_2 plants exhibited lower *rbcS* transcript amounts than their ambient-CO_2 counterparts. Furthermore, at elevated [CO_2], increasing growth temperatures resulted in a linear and substantial decrease in transcript abundance, with only one-third the amount at 44 °C as compared to 28 °C (Figure 2 B and C). For ambient CO_2-grown plants, the decrease in *rbcS* transcript amount between 28 and 40 °C was also substantial (45 %).

At 28 °C there was no effect of CO_2 enrichment on the total amount of soluble protein in the leaf (Figure 3). There did appear, however, to be some interactive effects between high growth [CO_2] and temperature because the soluble protein almost doubled in the high-CO_2 treatments as temperatures rose to 40 °C, whereas at ambient [CO_2] it only increased by 20 %. Although beyond 40 °C there was a sharp decline in soluble protein, at 48 °C it was still only 16 % less than at 28 °C. The responses of total soluble protein (Figure 3) to increases in [CO_2] and temperature were much greater than those of Rubisco protein (Figure 2 B), though the overall trends were similar.

The total Chl content per unit leaf area was also markedly affected by both [CO_2] and temperature regimes (Figure 3). Plants at elevated [CO_2] had from 25 to 40 % more Chl than those at ambient [CO_2]. Likewise, temperature increases up to 40 °C resulted in higher leaf Chl content, by as much as 50 % in the elevated [CO_2] treatment, but higher temperatures caused it to decline. However, even at 48 °C the Chl content was only 24 % less than at 28 °C.

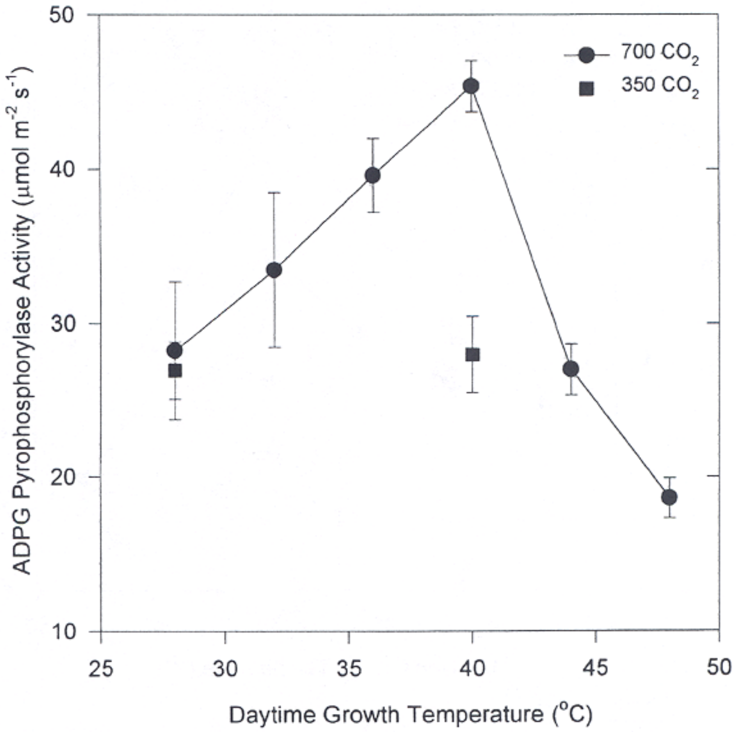

Figure 4. Activity of ADPG pyrophosphorylase in midday-sampled leaves of soybean plants grown under 350 and 700 μmol CO_2 mol^{-1} and various temperature regimes. Uppermost fully expanded leaves were harvested for analysis 48 DAP. Enzyme activity was expressed on a leaf area basis. Each data point represents the mean (with SE bar) of 3 determinations.

Activities of key enzymes involved in starch and sucrose metabolism were determined for soybean leaves sampled at predawn and midday. Since the overall patterns of ADPG pyrophosphorylase, SPS and invertase activities for predawn-sampled leaves were similar to those of midday-sampled leaves, only activities of the enzymes for leaves sampled at midday are shown. In elevated [CO_2], ADPG pyrophosphorylase activity for midday-sampled leaves increased almost linearly by 61 % with increasing growth temperature from 28 to 40 °C, but then declined; though at 48 °C it was still 66 % of the 28 °C rate (Figure 4). By contrast, under ambient [CO_2], there was essentially no difference in the activity of this enzyme for plants at growth temperatures of 28 and 40 °C. Consequently, the percentage CO_2 enhancement in ADPG pyrophosphorylase activity, which was only 5 % at 28 °C, increased to 62 % at 40 °C.

Figure 5 A shows the SPS activity for midday-sampled leaves. In the elevated-CO_2 plants, the V_{max} activity of SPS was greatest at 36 to 40 °C, but declined substantially at higher growth temperatures, much more so than that of ADPG pyrophosphorylase. For the ambient [CO_2] treatment, an increase in growth temperature from 28 to 40 °C had no effect on either the V_{max} or V_{limit} SPS activities (Figure 5 A). The SPS activity was higher in the elevated [CO_2] treatments, and the CO_2 enhancement effect rose with temperature. Thus, the V_{max} activity of SPS was 10 % higher in elevated [CO_2] at 28 °C, but 46 % greater at 40 °C.

As with other enzymes of carbohydrate metabolism examined in this study, acid and neutral invertase activities for midday-sampled leaves were greater (88 and 122 % respectively at 28 °C, and 39 and 46 % respectively at 40 °C) in leaves from the elevated, as opposed to the ambient, [CO_2] treatments (Figure 5 B). Regardless of growth treatment, acid invertase activity was three- to four-fold higher than the neutral form. Increasing growth temperatures up to 40 °C also increased the leaf invertase activities, but unlike SPS, the degree of CO_2 enhancement declined with a rise in growth temperature from 28 to 40 °C.

A doubling of growth [CO_2] substantially increased leaf starch and soluble sugars, and this appeared to be true throughout the diel cycle (Figure 6). In all cases carbohydrate amounts were greater at midday than at dawn. The predawn and midday starch content in leaves from elevated-CO_2 plants was 68 and 59 % higher respectively at 28 °C, and 374 and 164 % greater respectively at 40 °C, than their ambient-CO_2 counterparts (Figure 6 A). Similarly, contents of sucrose and hexoses (glucose and fructose) were also up to three-fold higher at elevated [CO_2] (Figure 6 B).

Unlike the rise in [CO_2], increasing growth temperatures reduced total starch contents of soybean leaves, especially during the night (Figure 6 A). Predawn and midday leaf starch contents in the ambient-CO_2 plants at 40 °C were only 11 and 52 % of those at 28 °C. At elevated [CO_2], a temperature rise from 28 to 40 °C had only a marginal effect on midday starch content, but predawn values declined by 68 %. At temperatures above 40 °C, day as well as night starch content was substantially reduced (Figure 6 A).

In contrast to the results for starch, midday sucrose in the high-CO_2 plants at 40 °C was almost double that at 28 °C, and though it declined at 48 °C the amount was similar to that at

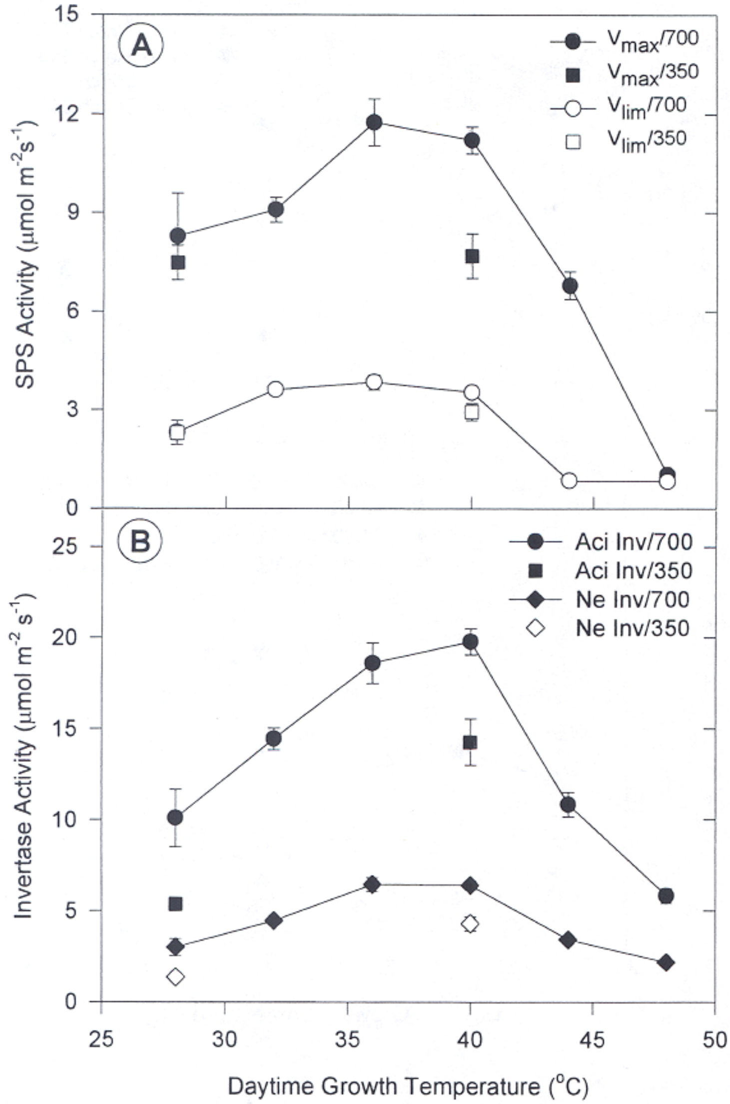

Figure 5. Activities of sucrose-P synthase (SPS) **(A)** and invertase (Inv) **(B)** in midday-sampled leaves of soybean plants grown under 350 and 700 µmol CO_2 mol^{-1} and various temperature regimes. Uppermost fully expanded leaves were harvested for analysis 48 DAP. Enzyme activities were expressed on a leaf area basis. SPS was measured under both saturating (V_{max}) and limiting (V_{lim}) substrate conditions. Invertase was assayed for acid (Aci) and neutral (Ne) Inv. Each data point represents the mean (with SE bar) of 3 determinations. Where no bar is visible, the SE is smaller than the symbol.

28 °C (Figure 6 B). Predawn sucrose values showed only a slight decline over the 28 to 48 °C range. Hexoses comprised the smallest fraction of the carbohydrates. At elevated [CO_2], hexoses were higher at midday than at predawn, and were greatest at 44 °C for both predawn and midday leaf sampling (Figure 6 B).

The net change in carbohydrates between dawn and midday was computed for each treatment by subtracting predawn from midday values (Figure 7 A and B). Between growth temperatures of 28 to 40 °C, the amount of starch accumulated during the morning hours rose dramatically, especially in the elevated [CO_2] treatment which exhibited over a four-fold gain. Beyond 40 °C there was a substantial decline, but the amount accumulated at 48 °C was still in excess of that at 28 °C. Temperature also influenced the elevated [CO_2] effect, such that starch accumulation at 28 °C was only 9 % more than in the ambient CO_2 treatment, whereas at 40 °C it was more than doubled.

As with starch, the morning accumulation of sucrose and hexoses was increased by elevated [CO_2], but especially by elevated growth temperatures, reaching a maximum at around 40 °C (Figure 7 B). Thus at 40 °C, sucrose accumulation was about three-fold higher at 700 than at 350 µmol CO_2 mol^{-1}. Once again, accumulations of sucrose and hexoses in elevated [CO_2] were greater at the supraoptimal temperature of 48 than at 28 °C.

At 48 DAP, area and dry weight of the uppermost, fully-expanded single leaflets in the elevated CO_2 treatment increased with increasing growth temperature and were greatest at 36 °C (Table 1). Although there were declines beyond 36 °C, leaf area and dry weight at 40 and 44 °C were still very comparable to the values at 32 °C. Even at 48 °C leaf area was still similar to that at 28 °C. The difference in leaf weight between 28 and 48 °C for the elevated CO_2 treatment was mostly due to a higher starch content at 28 °C growth (Figure 6 A). At 28 and 40 °C, leaf area and weight of the elevated-

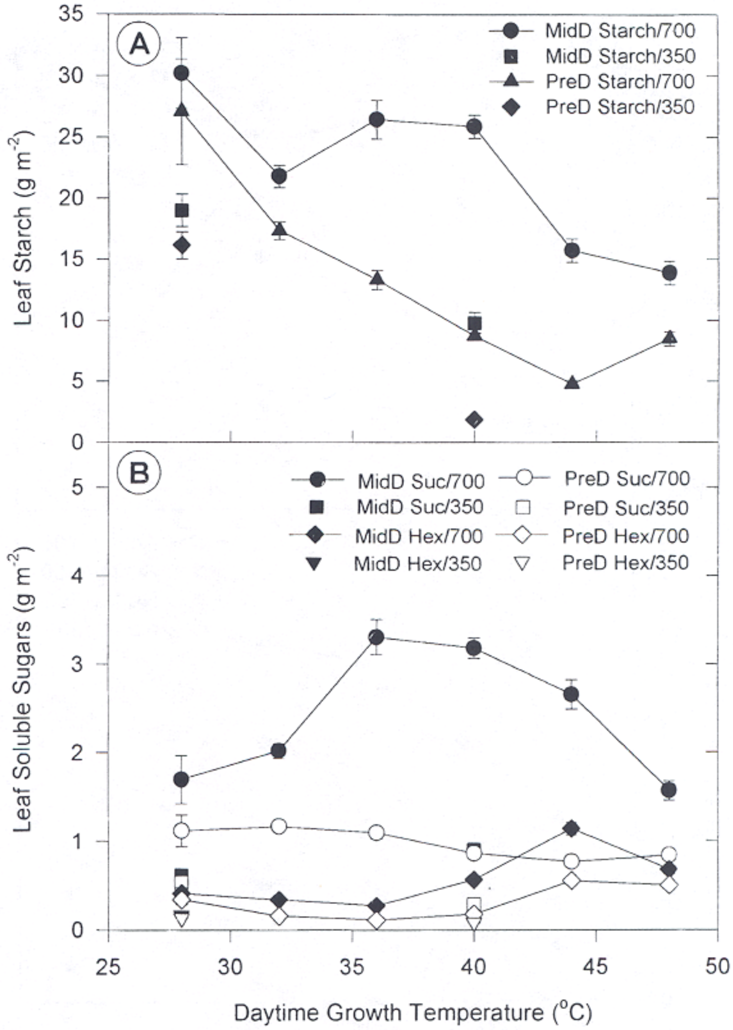

Figure 6. **(A)** Leaf starch and **(B)** leaf sucrose (Suc) and hexoses (Hex) in predawn (PreD)- and midday (MidD)-sampled leaves of soybean plants grown under 350 and 700 µmol CO_2 mol^{-1} and various temperature regimes. Uppermost fully expanded leaves were harvested for analysis 48 DAP. Contents of starch and soluble sugars were expressed on a leaf area basis. Each data point represents the mean (with SE bar) of 3 determinations. Where no bar is visible, the SE is smaller than the symbol.

CO_2 plants were greater than those of their counterparts grown at ambient [CO_2].

Discussion

Soybean leaf photosynthesis and carbohydrate metabolism under elevated [CO_2] remained relatively high even at growth temperatures above the values predicted by atmospheric general circulation models based on a doubling of atmospheric CO_2 (Wilson and Mitchell 1987, Hansen et al. 1988, Kattenberg et al. 1996). For instance, under elevated [CO_2], at 44/34 °C photosynthesis was only 15 % less than at 28/18 °C, and in comparison to ambient-CO_2 controls at 28/18 °C, was 17 % greater. The turnover rate of nonstructural carbohydrates, particularly starch and sucrose, may have played a major role. Although the total leaf starch contents at midday and predawn samplings were greater for soybeans grown at 28/18 °C under both ambient and elevated [CO_2] (Figure 6 A), the metabolism of starch was not efficient at this growth temperature regime. At 28/18 °C, most of the starch accumulating in the leaves for both CO_2 treatments was considered as "old" or "background" starch, since there was not much synthesis of "new" starch during the daylight hours. This was evident by the increased rate of net starch and sucrose gain (Figure 7). For soybean, nighttime temperatures may have been a critical environmental factor for the efficiency of starch catabolism. High night temperature could stimulate carbohydrate utilization, thus limiting the degree to which carbon accumulates. This might have very important implications in terms of reducing carbohydrate feedback inhibition of photosynthesis, as an increase in leaf carbohydrates has long been associated with an inhibition of leaf photosynthesis (Neals and Incoll 1968). For soybean in this study, a night temperature of 18 °C imposed a restriction on leaf starch decomposition during the evening, causing starch to accumulate, and this in turn negatively impacted leaf photosynthesis during the day. Additionally, visual leaf chlorotic symptoms were noticed for plants of both CO_2 treatments at 28/18 °C, also confirming less leaf chlorophyll content (Figure 3) as well

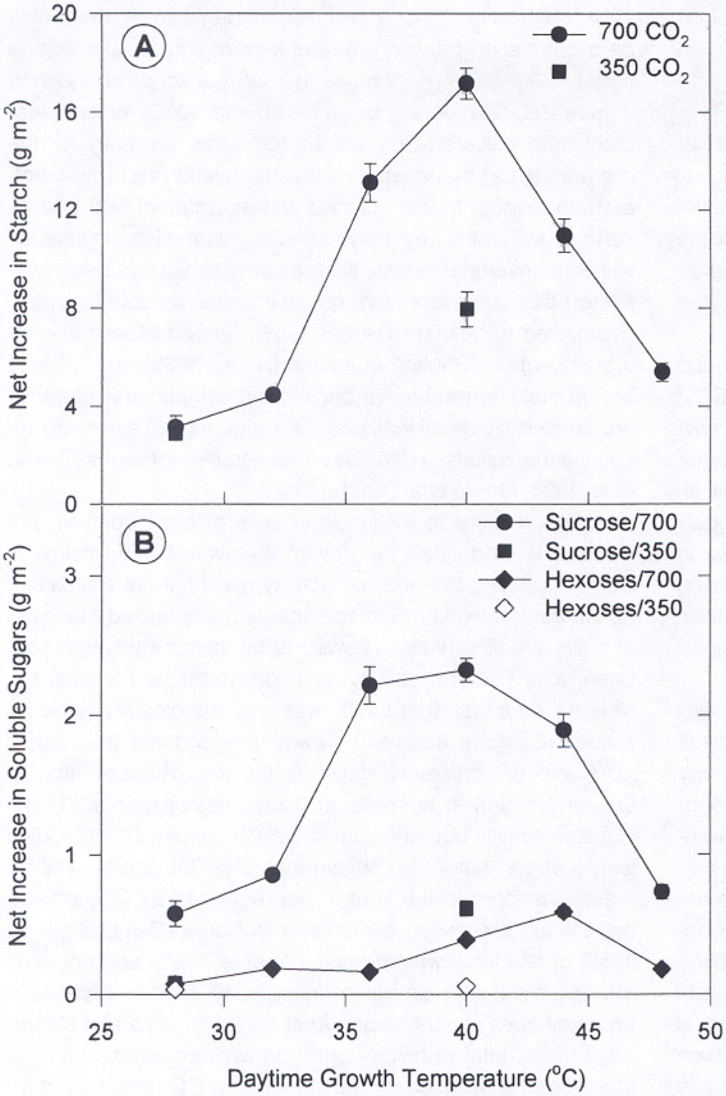

Figure 7. Net increases in starch **(A)** and soluble sugars (sucrose and hexoses) **(B)** in leaves of soybean plants grown under 350 and 700 μmol CO_2 mol^{-1} and various temperature regimes. Net increases in contents for each individual carbohydrate component of each treatment were computed from the data of Figures 6 A and B by subtracting predawn values from those of midday, and were expressed on a leaf area basis. Each data point represents the mean (with SE bar) of 3 determinations. Where no bar is visible, the SE is smaller than the symbol.

Table 1. Leaf area and dry weight of soybean plants grown at 350 and 700 μmol CO_2 mol^{-1} and under varying day/night maximum/minimum air temperature regimes. Uppermost, fully expanded leaflets were sampled at midday, 48 days after planting. Values are the mean ± standard error of 5 leaflets.

Temperature Regime (°C)	[CO_2] (μmol mol^{-1})	Area (cm^2 leaflet^{-1})	Dry Weight (mg leaflet^{-1})
28/18	350	42.1 ± 1.3	211.5 ± 17.8
	700	60.0 ± 1.6	378.3 ± 32.7
32/22	700	68.7 ± 5.2	386.9 ± 13.6
36/26	700	78.1 ± 5.8	486.0 ± 27.8
40/30	350	57.2 ± 3.8	261.0 ± 31.0
	700	64.6 ± 0.6	412.5 ± 7.4
44/34	700	67.2 ± 5.2	418.4 ± 15.3
48/38	700	62.4 ± 10.2	275.3 ± 33.4

as vegetative growth limitation (Pan 1996, Allen and Boote 2000) for this temperature treatment.

Under elevated growth [CO_2], soybean ADPG pyrophosphorylase activities increased with growth temperatures up to 40 °C and paralleled net increases in starch. In contrast, for the ambient-CO_2 plants, activities of ADPG pyrophosphorylase were similar at both 28 and 40 °C, although the net increase in starch at 40 °C was almost three-fold higher. At 40 °C, ADPG pyrophosphorylase activity of the elevated [CO_2] was 1.6-fold higher than that of the ambient control. By assuming that the net increase in starch was that which accumulated after about 6 h of morning sunlight exposure, the accumulation rate of starch at 40 °C growth was 2.2-fold greater at elevated than at ambient [CO_2], i.e., 2.9 g m^{-2} leaf area h^{-1} at elevated [CO_2] vs. 1.3 g m^{-2} leaf area h^{-1} at ambient [CO_2].

As with ADPG pyrophosphorylase, SPS activity of the elevated-CO_2 plants also increased with increased growth temperature up to 36–40 °C, while that of the ambient-CO_2 plants was similar at both 28 and 40 °C. In addition, activities of invertase for the elevated-CO_2 plants were much higher than those of the ambient-CO_2 plants, even though plants at both [CO_2] had higher enzyme activity at 40 °C than at 28 °C. In soybean, although activity of acid invertase exceeds that of neutral invertase, it is still unclear whether a discrete neutral enzyme with low activity is present, or neutral invertase activity is a residual activity of the acid form (Huber 1989). For the elevated-CO_2 soybeans, changes in activities of SPS and invertase with increasing growth temperatures also reflected changes in net increases in sucrose and hexoses.

Both long-term growth [CO_2] and temperatures influenced the levels of *rbcS* mRNA in soybean. Elevated growth [CO_2] induced a reduction of *rbcS* transcript abundance in soybean (this study), and has been shown to do so in other crops (Webber et al. 1994, Gesch et al. 1998, Moore et al. 1998, 1999, Vu et al. 1999). With respect to growth temperature, this study is the first to show substantial declines in *rbcS* transcript abundance over a wide range of increasing growth temperatures. In soybean cell cultures, short-term heat shock treatment at 40 °C for 2 h also drastically reduced the Rubisco *rbcS* transcript level (Vierling and Key, 1985).

Transcriptional rate and posttranscriptional stability and turnover of mRNA are factors that may have influenced the decline in *rbcS* transcripts observed for soybean in this study with increasing growth temperatures. The effect that long-term elevated growth temperature may have had on transcription and turnover rate of *rbcS* messages cannot be discerned from this study. However, the fact that steady state levels of soybean *rbcS* mRNA dramatically declined with increasing growth temperature, despite no specific association with changes in Rubisco protein content up to 40 °C, indicates that transcriptional rate and/or posttranscriptional events likely played a role in its decrease. Lack of correlation between *rbcS* expression and Rubisco protein content has been reported for several plant species grown under elevated [CO_2] (Moore et al. 1998). In yeast (*Saccharomyces cerevisiae*), a quantitative comparison of mRNA transcript and protein expression levels for a large number of genes also shows that the resultant correlation is insufficient for prediction of protein levels from mRNA transcript abundance (Gygi et al. 1999).

Levels of soluble sugars in plant cells have been shown to influence the expression of several genes coding for key photosynthetic enzymes (Jang and Sheen 1994, Koch 1996). It has also been suggested that plant growth under temperature stress, in addition to elevated [CO_2], may lead to increases in leaf carbohydrate levels which may then repress the expression of genes encoding for Rubisco (Webber et al. 1994). Recent evidence, however, indicates that the sucrose cycling (i.e., sucrose hydrolysis and/or transport) and hexokinase-mediated sugar sensing may directly play an integral role in sugar-mediated regulation of the expression of photosynthetic genes (Jang and Sheen 1997, Smeekens and Rook 1997, Cheng et al. 1998, Moore et al. 1998, 1999, Smeekens 1998, Pego et al. 2000). For soybean in this study, there may be a correlation between the net increase in soluble sugars (Figure 7 B) and the decrease in *rbcS* transcript abundance (Figure 2 B), particularly between 28 and 40 °C, for both ambient- and elevated-CO_2 treatments. More evidently for the elevated [CO_2] treatment, this inverse relationship was greatest with respect to leaf sucrose and activities of SPS and invertase, two of the key enzymes in its metabolism. In species with high invertase activity such as soybean, it has been postulated that sucrose which moves into the vacuole is rapidly hydrolyzed to hexoses (Huber 1989). Since hexoses in general do not accumulate in leaves (Huber 1989), the glucose and fructose formed move back to the cytoplasm where they are sensed by hexokinases, thus triggering a repression response that results in decreased *rbcS* transcript levels (Cheng et al. 1998, Moore et al. 1999).

Although there is evidence in several plant species that Rubisco is modulated by growth at elevated CO_2, claims of down-regulating the enzyme activity need careful evaluation, as the basis on which Rubisco activity is expressed may vary or nullify the observation (Bowes 1993). In this study with soybean, total Rubisco activity for midday-sampled leaves, expressed on a leaf area basis, was only marginally altered by growth [CO_2]. In addition, growth temperatures from 28 to 40 °C did not markedly influence the total Rubisco activity. Only when growth temperatures were higher than 40 °C did Rubisco activity become substantially inhibited. Other studies with soybean have also shown that total Rubisco activity for leaves sampled in the light is not regulated by CO_2 enrichment when expressed either on a leaf area (Campbell et al. 1988) or leaf fresh weight basis (Vu et al. 1997), and not at all affected by growth temperatures up to 40 °C when expressed on a leaf fresh weight basis (Vu et al. 1997). On a leaf chlorophyll basis, total Rubisco activity in midday-sampled leaves of soybean is reduced by growth under a CO_2 enrichment regime (Vu et al. 1983, 1987, 1989). However, this decrease in Rubisco activity results as an increase in leaf chlorophyll content of the CO_2-enriched soybean plants (Vu et al. 1989, Figure 3 of this study).

The results from this study indicate that soybeans grown for a season under a double-ambient CO_2 atmosphere and a wide range of temperatures exceeded their ambient CO_2-grown counterparts in most aspects of photosynthetic capacity and carbon metabolism. Leaf CER of soybeans grown at elevated [CO_2] doubled that of ambient-CO_2 plants at 40 °C, and even at 48 °C was still as high as that for ambient-CO_2 plants at 28 °C. Similarly, activities of the key enzymes for starch and sucrose metabolism at 40 °C were greater under elevated than at ambient [CO_2]. With increasing growth temperature, soybean photosynthesis and carbohydrate metabolism performed well at maximum daytime temperature up to 40 °C. Measurements of above-ground biomass at final harvest for plants in this experiment also indicate that soybean vegetative growth was quite tolerant to high temperatures up to even 44 °C at elevated [CO_2], although the 36 °C treatment appeared to be the optimum growth temperature for individual leaflets (Table 1) and plant biomass accumulation (Pan 1996, Allen and Boote 2000). However, it should be noted that VPD between the highest and lowest temperature treatment was substantial and may have influenced the apparent temperature-optimum for plant growth. But in "real-world" conditions higher air temperatures will be associated with higher VPDs. Thus, in the absence of other climatic stresses, soybean photosynthesis would perform well under rising CO_2 and temperature conditions predicted for the 21st century. However, one must be cautious in extrapolating high temperature tolerance of soybean photosynthesis and vegetative performance to economic seed yield production (Pan 1996, Allen and Boote 2000).

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